A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN MEDIA OF VARIED COMPOSITION

STUDIES IN ACID-FAST BACTERIA. IX*

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In the previous articles it has been shown that broth cultures of various acid-fast bacteria exhibit lipolytic activity; that this lipolytic activity is also demonstrable in the autolyzed bacteria from the same media; and that the lipase in solution in the broth and in the bodies of the bacteria grown in this broth is thermostabile. Furthermore, this lipase, irrespective of the composition of the medium in which it is produced, acts on various esters and glycerids. The extent of lipase activity observed, both in culture media and in autolyzed bacteria taken from these media, appears to vary with the luxuriance of the growth of the organisms. This observation, however, appears to apply more strictly to the filtered broth cultures than to the autolyzed bacteria obtained from these cultures, and the question arises: Is this lipolytic activity proportionate to the amount of autolysis which the bacteria undergo in the media, or does this lipolytic activity vary with the vegetative activity (metabolism) of the organisms? The solution of this question possesses more than academic interest, for it is conceivable that an active exolipase might play a not unimportant part in the development of the tubercle bacillus in the human body.

If the former possibility alone were realized, tubercle bacilli should exhibit lipolytic activity more or less proportionate to their autolysis; whereas, if the latter possibility alone (or associated with the former possibility) were involved, there would be a rough parallelism between the amount of lipolysis demonstrable in culture and the extent of vegetative activity of the organisms themselves in the same culture, provided appropriate media were used. That is to say, if the tubercle bacillus produces an exolipase, it is probable that the curves of lipolytic activity and vegetative activity would reach their maxima more or less synchronously.

* Received for publication July 27, 1914.

TABLE 1

METABOLISM OF TUBERCLE BACILLI IN MEDIUMS A, B, AND C

Medium	Days	Dextrose					Mannite					Glycerin				
		Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH
A	14	—0.10	—4.2	—10.0	0.25	......	—0.20	—4.2	—10.0	0.30	......	0.00	—4.2	—10.0	0.40	....
	21	—0.15	—4.2	—10.0	0.40	0.40	—0.40	—4.2	—10.0	0.55	0.40	+0.10	—4.9	11.66	0.30	0.45
	28	—0.05	—2.8	6.56	0.35	0.25	—0.20	—2.1	—5.0	0.35	0.35	+0.30	—4.2	10.0	0.25	0.30
B	14	—0.05	+4.9	9.0	0.20	......	—0.20	+16.1	29.5	0.25	....	0.00	+4.2	15.4	0.30	....
	21	—0.05	+13.3	24.4	0.50	0.10	—0.20	+20.3	37.2	0.30	0.05	+0.10	+7.0	12.8	0.40	0.00
	28	—0.05	+23.8	43.5	0.75	0.15	0.00	+21.0	38.4	0.65	0.20	+0.10	+12.6	23.1	0.60	0.35
C	14	—0.20	+9.1	10.2	0.45	......	0.00	+23.1	25.8	0.60	....	—0.50	+4.2	4.62	0.55	....
	21	—0.20	+18.9	21.1	0.70	0.75	0.00	+23.1	25.8	1.00	0.60	—0.30	+14.0	15.4	0.50	0.50
	28	0.00	+16.1	18.0	0.45	0.20	—0.20	+26.6	29.7	0.90	0.45	+0.10	+17.5	19.3	0.85	0.55

* Figures expressed as NH_3 per 100 c.c. (Medium A) represent the total soluble nitrogen of the clear fluid underlying the bacterial growth: full details in text.

TABLE 2

GROWTH OF TUBERCLE BACILLI IN MEDIUM D

Bacillus Tuber-culosis	Days	Dextrose					Mannite					Glycerin				
		Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH_3mg. Increase per 100 c.c. Media	NH_3 / Total N Per-cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH
WI	21	+0.40	23.8	32.1	0.50	....	+0.20	30.8	46.8	0.60	....	+0.30	9.8	15.2	0.60	....
	28	+0.30	36.4	49.1	0.70	0.65	+0.20	39.9	60.3	0.95	0.65	+0.30	28.0	43.4	0.60	0.55
	35	+0.20	30.8	41.5	0.45	0.15	+0.30	30.8	46.8	0.70	0.15	0.00	30.8	47.8	0.45	0.25
	42	+0.10	34.3	46.3	0.50	0.15	+0.10	31.5	48.0	0.75	0.10	+0.10	33.6	52.1	0.40	0.10
WII	21	0.00	14.0	18.9	0.50	....	+0.20	31 5	48.0	0.70	....	+0.20	14.0	21.8	0.60	....
	28	+0.20	36.0	49.1	0.65	0.65	+0.10	37.8	57.5	0.95	0.55	+0.20	27.3	42.4	0.55	0.80
	35	+0.30	31.5	42.4	0.60	0.30	0.00	30.8	46.8	0.70	0.10	+0.10	32.2	50.0	0.45	0.40
	42	+0.30	33.6	45.3	0.50	0.10	+0.10	33.6	51.1	0.60	0.10	+0.10	35.0	54.4	0.40	0.10
597I	21	+0.50	18.2	24.5	0.50	....	+0.10	30.1	45.8	0.60	....	+0.20	23.8	37.6	0.50	....
	28	+0.50	32.9	44.3	0.70	0.40	+0.10	39.9	60.3	1.10	0.50	+0.20	25.2	39.3	0.60	0.90
	35	+0.50	30.1	40.5	0.45	0.25	+0.10	32.2	49.0	1.45	0.20	+0.20	28.0	43.4	0.40	0.20
	42	+0.20	32.2	43.4	0.40	0.25	0.00	31.5	48.0	0.55	0.20	+0.40	31.5	49.7	0.35	0.15
597II	21	+0.30	17.5	23.6	0.50	....	+0.10	30.1	45.8	0.70	....	+0.30	10.5	16.3	0.55	....
	28	+0.30	31.5	42.4	0.75	0.55	+0.10	39.9	60.3	1.05	0.65	+0.30	25.2	39.3	0.40	0.50
	35	+0.30	31.5	42.4	0.45	0.20	+0.10	31.5	48.0	0.25	0.20	+0.10	23.8	37.6	0.25	0.25
	42	+0.30	31.5	42.4	0.60	0.15	+0.20	31.5	48.0	0.65	0.15	+0.30	32.2	50.0	0.30	0.20

With these two possibilities in view, the following experiments were undertaken to demonstrate the relationship, if such exists, between the lipolytic activity of cultures of certain acid-fast organisms and the metabolism of these organisms, as measured by ammonia formation and a change in reaction. In the present communication the metabolism of two rapidly growing human tubercle bacilli is considered. The procedures followed are precisely those described in previous communications where the full details are given.

Briefly, the metabolism of the tubercle bacilli (vegetative activity) was measured by the changes in ammonia content of the medium, which indicates the action of the organisms on the protein constituents, and the changes in

TABLE 3
GROWTH OF TUBERCLE BACILLI IN MEDIUM E

Bacillus Tuberculosis	Days	Plain Broth (a)				Plain Broth (b)			
		Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{Total\ N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH	Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{Total\ N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH
597	3	—0.50	1.4	1.45	0.15	—1.30	11.9	5.30	0.20
	7	—1.30	23.8	17.9	0.10	—1.70	49.7	22.2	0.05
	14	—1.20	37.8	28.4	0.10	—1.80	64.4	28.7	0.40
	21	—1.30	35.0	26.3	0.15	—1.90	60.2	26.9	0.15
	28	—1.30	28.7	21.6	0.30	—1.90	52.5	23.4	0.45
W	3	—0.90	4.2	3.15	0.10	—1.40	11.9	5.32	0.20
	7	—1.30	29.4	22.1	0.10	—1.90	52.5	23.5	0.05
	14	—1.10	38.5	29.0	0.15	—1.70	65.1	29.0	0.45
	21	—1.40	35.0	26.3	0.10	—2.00	60.9	27.2	0.45
	28	—1.10	28.7	21.6	0.35	—1.70	43.8	21.5	0.50

reaction to phenolphthalein. The lipase activity was measured according to the method described previously. It consisted, essentially, in suspending 1 c.c. of the bacteria-free filtrate of the various cultures in freshly boiled, distilled water, adding 0.25 c.c. of ethylbutyrate and 0.5 c.c. of toluene, and incubating at 37 C. together with appropriate controls. The increase in acidity in terms of N/50 NaOH is taken as a measure of the lipolytic activity of the culture for the period of incubation mentioned.

The organisms have been studied in media of extremely simple composition, and through successive degrees of complexity to ordinary nutrient broths with various sources of carbon, as follows:

Medium A (Table 1).—$(NH_4)_2\ HPO_4$, 4 gm., and NaCl, 5 gm., in 1,000 c.c. redistilled water with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

It will be observed that the determination "Ammonia" (Table 1) is in reality the determination of the total soluble nitrogen in this medium, for all

the nitrogen of $(NH_4)_2HPO_4$ is measured by the Folin Air Current Method of ammonia estimation. The decrease in "ammonia," therefore, observed during the second and third weeks of incubation, represents the amount of nitrogen appropriated by the bacteria as they increased in numbers. The reappearance of nitrogen, observed at the end of the fourth week in the filtrates of the cultures in this medium (associated with a decrease in lipolytic activity of the solution), is probably to be regarded as an indication of autolysis of the bacteria with the liberation from them of nitrogenous substances which again pass into solution, and are recovered as "ammonia."

Medium B (Table 1).—Asparagin, 4 gm., Na_2HPO_4, 2 gm., and NaCl, 5 gm., in redistilled water, 1,000 c.c., with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

Medium C (Table 1).—Asparagin, 2 gm., $(NH_4)_2HPO_4$, 2 gm., Na_2HPO_4, 1 gm., NaCl, 5 gm. in 1,000 c.c. of redistilled water.

TABLE 3—(*Continued*)
GROWTH OF TUBERCLE BACILLI IN MEDIUM E

Bacillus Tuberculosis	Days	Dextrose Broth (a)				Glycerin Broth (b)			
		Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH	Phenolphthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethylbutyrate c.c. N/50 NaOH
597	3	—1.20	5.6	4.00	0.15	—0.50	3.5	2.63	0.10
	7	—1.40	1.40	1.00	0.10	—0.80	1.4	1.05	0.15
	14	—1.40	17.5	12.50	0.10	—0.80	—2.8	—2.1	0.15
	21	—1.50	23.1	16.50	0.15	—0.80	—2.8	—2.1	0.20
	28	—1.40	23.8	17.0	0.50	—0.90	—2.8	—2.1	0.10
W	3	—1.30	6.3	4.50	0.15	—0.60	2.1	1.58	0.15
	7	—1.20	6.3	4.50	0.30	—0.60	3.5	2.63	0.10
	14	—1.50	29.4	21.0	0.95	—0.80	—0.70	—0.52	0.15
	21	—1.50	23.1	16.5	0.95	—0.70	—2.1	—1.58	0.15
	28	—1.40	23.8	17.0	0.90	—0.90	—2.8	—2.1	0.10

Medium D (Table 2).—Asparagin, 4 gm., $(NH_4)_2HPO_4$, 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water.

Medium E (Table 3).—Fairchild's peptone (extracted with ether, alcohol, acetone, and petroleum ether to remove lipoids and fat in Medium E[a] and unextracted in Medium E[b]), 5 gm., Na_2HPO_4, 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water, with 1 percent dextrose, or 3 percent glycerin.

Medium F (Table 4).—Regulation sugar-free meat-juice-peptone broth with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

As shown in Table 1, in the ammonium phosphate medium (A), the maximum nitrogen metabolism was reached on the twenty-first day, at which time the organisms had removed 10 percent of the total medium from solution; this nitrogen was probably incorporated in their bodies. Lipase activity is also maximum at this time. By the end of the twenty-eighth day, autolysis of the bacteria was well under

way (shown by the reappearance of some of the nitrogen in solution) and lipolytic activity had diminished somewhat.

In the asparagin medium (B) there is steady increase in metabolism in the dextrose, mannite, and glycerin modifications, respectively, associated with a progressive increase in lipase activity. This experiment was not carried far enough to show the recession in metabolism, but other studies with the same media indicate that at the end of the fourth week there is usually a recession of metabolism.

In the ammonium phosphate-asparagin medium (C) the maximum of metabolism and lipolytic activities is reached on the twenty-first day, except in the glycerin modification.

TABLE 4
GROWTH OF TUBERCLE BACILLUS W IN MEDIUM F

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.10	0.00	0.00	0.15	—0.50	—0.70	—0.22	0.15
3	—0.30	0.70	0.22	0.20	—1.10	—2.80	0.88	0.55
6	—0.90	2.8	0.88	1.90	—1.60	1.40	0.44	0.75
10	—1.40	16.8	5.22	1.60	—1.60	14.70	4.57	2.50
15	—1.70	22.4	6.96	1.65	—2.10	28.7	8.60	1.75
21	—1.70	26.6	8.30	1.90	—1.80	34.3	10.65	2.60
28	—1.10	14.0	4.40	1.40	—1.70	26.6	8.30	1.40
36	—2.10	5.6	1.74	1.25	—2.40	9.8	3.04	1.05
43	—1.60	0.00	0.00	1.20	—2.50	7.0	2.17	1.10
51	—1.50	—2.8	—0.87	1.15	—2.10	—5.6	—1.74	1.00

The general parallelism between metabolism and lipolytic activity (Medium D) is clearly set forth in Table 2.

Medium E (a) consists of Fairchild's peptone extracted to remove all fats and lipoids. Growth was fairly luxuriant, but lipase activity is surprisingly low (Table 3). No explanation for this disparity can be advanced; certain other peculiarities—the production of a progressively alkaline reaction in glycerin and unusual products of growth—suggest that this peptone medium is acted on differently by the tubercle bacillus than the regulation meat-juice peptone media.

Medium F consists of regulation meat-juice-peptone broth, plain and with dextrose, mannite, or glycerin. The observations (Table 4) have been continued for fifty-one days; the maximal proteolytic activity is reached by the third week, at which time lipolytic activity appears

to be at its height. There is a great diminution in the ammonia content of the media after this time; at the end of fifty-one days' incubation there is actually less than at the start. The lipase activity declines considerably after three weeks, but not proportionately to the ammonia.

The recession of ammonia appears to be associated with autolysis of the bacteria, and in this connection the experiments of Lockemann[1] and Möllers[2] are suggestive. Lockemann showed that the weight of tubercle bacilli grown in glycerin broth increased steadily to a maximum, then diminished somewhat; and Möllers' observations would indicate that the antigenic content of the same culture paralleled the weight curve of Lockemann. It is a striking coincidence to compare

TABLE 4—(*Continued*)
GROWTH OF TUBERCLE BACILLUS W IN MEDIUM F

	Mannite Broth				Glycerin Broth			
Days	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH_3 mg. Increase per 100 c.c. Broth	$\frac{NH_3}{\text{Total } N_2}$ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.20	0.00	0.00	0.00	0.00	—1.4	—0.44	0.05
3	—0.40	0.70	0.22	0.40	—0.30	1.4	0.44	0.30
6	—0.80	6.3	1.95	1.85	—0.70	4.9	1.21	2.05
10	—0.70	16.8	5.22	2.20	—0.60	8.4	2.61	2.45
15	—0.80	28.7	8.90	1.80	—0.70	18.2	5.65	2.30
21	—0.70	29.4	9.12	1.65	—0.30	18.9	5.87	3.80
28	—0.60	25.9	8.05	1.45	+0.10	18.9	5.87	1.35
36	—0.60	25.2	7.83	1.30	—0.30	16.8	5.22	1.15
43	—0.50	21.7	6.74	1.35	0.00	11.2	3.48	1.25
51	—0.50	8.4	2.60	1.40	—0.20	—0.70	—0.21	1.15

these curves with the similar curves of metabolic and lipolytic activity, and the possibility suggests itself that these phenomena are closely related, if not identical, in origin.

The lipase curve, following the curve of proteolysis to its maximum, but diminishing far less rapidly during the period of recession, speaks strongly in favor of the view advanced in a previous article, that the lipase is an exoferment, in part at least. Otherwise lipase activity should increase progressively with autolysis, unless its activity is inhibited by the accumulation of products of its own production.

It was a point of some interest to determine just how much nitrogen is contained in the bacterial cells at the end of fifty-one days' incuba-

1. Veröffentlichungen der Robert Koch, Stiftung zur Bekämpfung der Tuberculose, 1914, 10, p. 21.
2. Ibid., p. 56.

tion. This was readily accomplished by comparing the total soluble nitrogen in media after fifty-one days' growth with the initial total nitrogen of media of the same composition inoculated under parallel conditions. The tabulated results show clearly that between 21 and 32 percent of the initial nitrogen is not in solution, and the logical inference is that this "lost" nitrogen is retained in the bodies of the bacteria.

Bacillus Tuberculosis W	Plain	Dextrose	Mannite	Glycerin
Initial total N_2—mg.	322	322	322	322
Final total N_2—mg.	231	217	224	252
Loss total N_2—mg.	91	105	98	70
Percent loss*	28.3	32.6	30.4	21.7

*The percentage of nitrogen not in solution, but incorporated in the bodies of the bacteria which had grown in 51 days.

There is a noteworthy parallelism between the curve of vegetative activity, as measured by ammonia formation, and the curve of lipolytic activity, as measured by the changes in acidity in all media. The period of greatest ammonia production, which is assumed to mark the period of maximum vegetative activity, coincides definitely with the period of greatest lipolytic activity; and the recession of ammonia, which has been commented on in the previous communications, appears to be associated with a decrease in the lipolytic activity of the cultures.

CONCLUSIONS

The period of maximum vegetative activity of broth cultures of certain avirulent, rapidly growing tubercle bacilli, as measured by ammonia formation (proteolysis), appears to coincide with the period of maximum lipolytic content of these cultures, as measured by their action on ethylbutyrate.

Both ammonia production and lipolytic activity are extremely slight during the first day's growth of these organisms, and increase, roughly, proportionately to their respective maxima.

There is a noteworthy recession of both factors after this maximum is reached.

These experiments appear to warrant the assumption that the organisms studied excrete a soluble, active lipase during the period of active development; for if autolysis alone were responsible for the lipolytic activity observed in these cultures, it should increase as autolysis proceeds.